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Protocols

Target Region Amplification Polymorphism: A Novel Marker Technique for Plant Genotyping

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Abstract. The advent of large-scale DNA sequencing technology has generated a tremendous amount of sequence information for many important organisms. We have developed a rapid and efficient PCR-based technique, which uses bioinformatics tools and expressed sequence tag (EST) database information to generate polymorphic markers around targeted candidate gene sequences. This target region amplification polymorphism (TRAP) technique uses 2 primers of 18 nucleotides to generate markers. One of the primers, the fixed primer, is designed from the targeted EST sequence in the database; the second primer, the arbitrary primer, is an arbitrary sequence with either an AT- or GC-rich core to anneal with an intron or exon, respectively. PCR amplification is run for the first 5 cycles with an annealing temperature of 35°C, followed by 35 cycles with an annealing temperature of 50°C. For different plant species, each PCR reaction can generate as many as 50 scorable fragments with sizes ranging from 50-900 bp when separated on a 6.5% polyacrylamide sequencing gel. The TRAP technique should be useful in genotyping germplasm collections and in tagging genes governing desirable agronomic traits of crop plants.

Key words: EST, genotyping, *Helianthus annuus*, PCR, target region amplification polymorphism

Abbreviations: EST, expressed sequence tag; nt, nucleotide; PCR, polymerase chain reaction; TRAP, target region amplification polymorphism.

Introduction

Molecular markers play an important role in the structural and functional genomics of animal, plant, and microbial species. Two of the most popular marker techniques are random amplified polymorphic DNA (RAPD) (Williams et al., 1990) and amplified fragment length polymorphism (AFLP) (Vos et al., 1995). Recently, Li and Quiros (2001) published a new molecular marker technique called sequence-related amplification polymorphism (SRAP), which uses pairs of primers with AT- or GC-rich cores to amplify intragenic fragments for polymorphism detection. The common feature of these techniques is that multiple fragments can be generated in a single PCR reaction, making them more efficient.

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However, these techniques use no prior sequence information, and the markers generated are randomly distributed across the genome. They are used jointly with bulked segregant analysis (BSA) (Michelmore et al., 1991) to screen for markers linked to desirable agronomic traits, such as disease resistance. Another useful and reliable marker class is the simple sequence repeat (SSR), or microsatellite marker, which uses known DNA sequence information to produce highly polymorphic and allele-specific markers but is limited in number for high-density map construction (Gupta and Varshney, 2000).

The advent of high-throughput sequencing technology has generated abundant information on DNA sequences for the genomes of many plant species. This includes the completion of the draft of the whole genome sequences for the model plant *Arabidopsis thaliana* in 2000 (The *Arabidopsis* Genome Initiative, 2000) and for rice, one of the most important food crops, in 2002 (Goff et al., 2002; Yu et al., 2002). In addition, the ESTs of other important crop species have been generated, and powerful bioinformatics tools have annotated thousands of sequences as putative functional genes. The task of bridging this DNA sequence information with particular phenotypes relies on molecular markers. Consequently, there is a strong demand for better marker techniques to better utilize the existing sequence information. Here we report a rapid and efficient PCR-based TRAP technique, which uses bioinformatics tools and the EST database information to generate polymorphic markers around targeted putative candidate gene sequences.

Materials and Methods

DNA preparation

Sunflower (*Helianthus annuus* L.) breeding lines were used in the development and refinement of the TRAP protocol. Seeds were germinated in the laboratory culture chamber for DNA isolation. Total DNA was isolated from young leaf tissue of sunflower seedlings using the DNeasy Plant Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. DNA concentrations were determined with a DU7400 spectrophotometer (Beckman Coulter) and adjusted to 30-50 ng/μL for PCR amplification.

Primer design, fixed primers

The fixed primer was constructed according to the following procedure: (1) identify the sequence of interest from the sunflower EST database (http://cgpdb.ucdavis.edu/database/php_my_admin/php_my_admin.php) (Michelmore, personal communication); (2) insert the sequence into the input window of the Web-based PCR primer designing program, Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) (Rozen and Skaletsky, 2000); (3) set the primer optimum size, maximum size, and minimum size to 18 nt; (4) set the primer optimum T_m, maximum $T_{m,}$ and minimum T_m to 53°C, 55°C, and 50°C, respectively; and (5) use one of the primers picked by the program.

Primer design, arbitrary primers

We adapted the concept of Li and Quiros (2001) to design the arbitrary primers. Three principles were considered in the construction of each random primer: (1) the selective nucleotides, 3-4 nt at the 3′ end; (2) the core, 4-6 nt with AT- or GC-rich regions; and (3) the filler sequences that make the 5′ end. We upheld the general principles of PCR primer design, such as the avoidance of self-complementarity and the maintenance of GC content (40-60%) for proper melting temperature of primers and retention of their correct internal stability. Sequences of the arbitrary primers used in the current study were provided by Li and Quiros (personal communication). Random primers are 3′ end-labeled with IR dye 700 or IR dye 800 for autodetection of the amplified fragment with the NEN Global DNA Sequencer (Li-Cor Biosciences, Lincoln, NE) equipped with the SAGA software. Labeled primers were synthesized by Li-Cor Biosciences or MWG Biotech (MWG Biotech Inc., High Point, NC).

PCR amplifications

PCR was conducted with a final reaction volume of 15 µL in 96-well microtiter plates in a GenAmper 9700 thermal cycler (Applied Biosystems, Foster City, CA) with the following components: 2 µL of the 30-50 ng/µL DNA sample, 1.5 µL of 10 × reaction buffer (Qiagen), 1.5 µL of 25 mM $MgCl_2$, 1 µL of 5 mM dNTPs, 3 pmol each of 700- and 800-IR dye–labeled arbitrary primers, 10 nmol of the fixed primer, and 1.5 U of *Taq* DNA polymerase (Qiagen). PCR was carried out by initially denaturing template DNA at 94°C for 2 min. We then performed 5 cycles at 94°C for 45 s, 35°C for 45 s, and 72°C for 1 min, followed by 35 cycles at 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min. The final extension step was at 72°C for 7 min.

Electrophoresis of amplified products

Upon completing the PCR cycles, 7 µL of 5 × loading dye (0.313 M Tris-HCl [pH 6.8] at 25°C, 10% SDS, 0.05% bromophenol blue, 50% glycerol) was added to the reaction mixture. A 1-µL aliquot was loaded onto a 6.5% polyacrylamide sequencing gel in a Li-Cor Global DNA Sequencer using protocols recommended by the manufacturer. Electrophoresis was conducted at 1500 v for 3.5 h, and the images were collected by were SAGA software.

Results and Discussion

The TRAP marker technique was initially developed with sunflower DNA samples. It has been tested by using DNA samples from different plant species, including rice, *Oryza sativa* L.; barley, *Hordeum vulgare* L.; wheat, *Triticum aestivum* L.; dry bean, *Phaseolus vulgaris* L.; and sugar beet, *Beta vulgaris*, L. (data not shown). TRAP primers amplified fragments with sizes ranging from 50-900 bp that were well separated in the 6.5% polyacrylamide gel (Figure 1). In most cases, 30-50 scorable fragments were amplified in each PCR reaction. Amplified fragments from each DNA sample could be classified into strong, intermediate, or weak categories on the basis of their intensity. This variation in

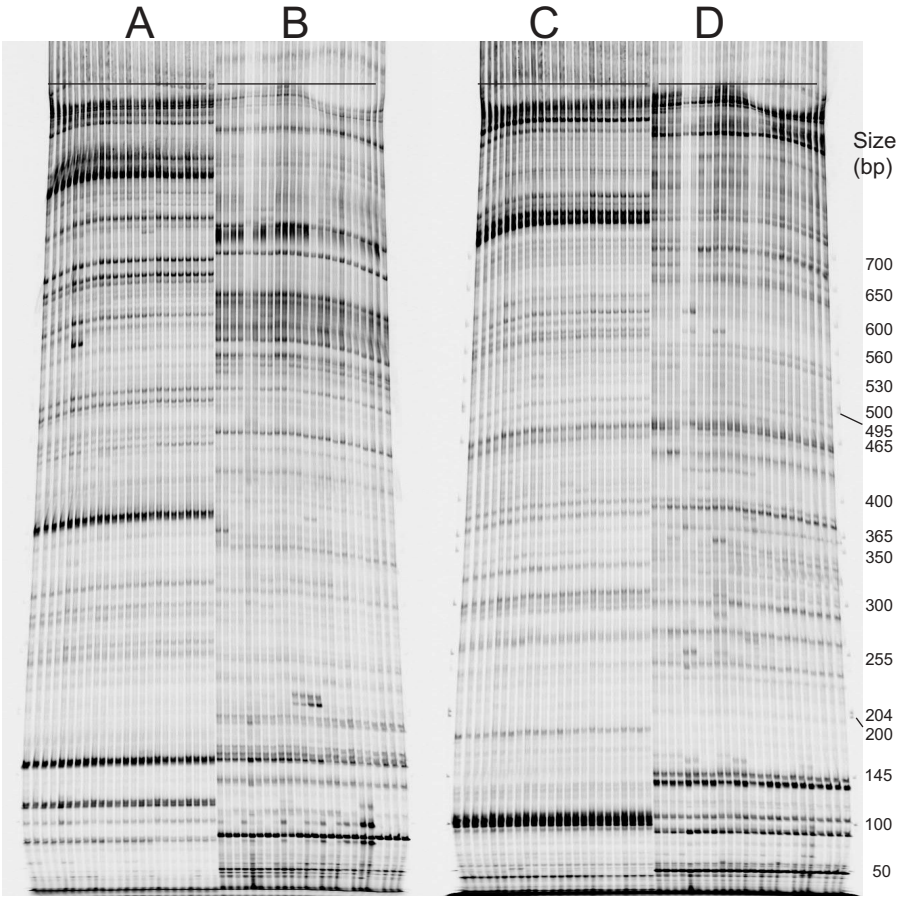

Figure 1. TRAP amplification patterns of 8 sunflower F1 hybrids (lanes 1-16, two DNA samples for each hybrid) and 8 maintainer lines (lanes 17-24, one DNA sample for each line) with 2 fixed and 4 arbitrary primers (panel A: fixed primer A21B09b [sequence: 5′-TGTCATTCAATTCGGTGC-3′] + arbitrary primer Ga5-800 [sequence: 5′-GGAACCAAACACATGAAGA-3′], panel B: B18I19b [sequence: 5′-CGTTTATTTCCTCGCCTC-3′] + Ga3-800 [sequence: 5′-TCATCTCAAACCATA-TACAC-3′], panel C: A21B09b+Sa4-700 [sequence: 5′-TTCTTCTTCCCTGGACACTT-3′], and panel D: B18I19b+Odd26-700 [sequence: 5′-CTATCTCTCGGGACCAAAC-3′]. The name of the fixed primer corresponds to the Sequence ID in the sunflower EST database. The 50- to 700-bp DNA size standard is from Li-Cor Biosciences.

amplification preference could be a result of the copy number of the fragment in the genome and/or the degree of complementarity of the end sequences to that of the primers. No obvious correlation between fragment size and fragment intensity was found.

Because the Li-Cor DNA Sequencer has 2 channels to detect fluorescent signals, the primers were labeled with 2 different dyes that emit fluorescence at

700 or 800 nm. Initially in our development of the TRAP method, we ran the PCR reactions with each dye-labeled primer separately and then mixed the amplified products prior to gel loading. Later, we found that 1 unlabeled fixed primer and 2 labeled arbitrary primers in the same PCR reaction produced similar results. This improvement increased the efficiency and reduced the cost per data point. Like RAPDs, TRAP markers are quick and easy to set up, and each reaction can produce a profile comparable to the AFLP technique. With perennial *Helianthus* species, we used 2 PCR reactions with 6 primers (2 fixed primers, targeting disease resistance gene sequences with leucine-rich repeats [LRR] or nucleotide binding site [NBS] regions, and 4 arbitrary primers) and generated more than 100 polymorphic fragments among the 16 species. A phylogenetic tree constructed with the TRAP marker data had the similar clustering as the classic taxonomic system, which is based on morphologic characteristics (Hu et al., 2003).

Reproducibility has been a concern for easily generated markers, such as RAPDs (Jones et al., 1997; Virk et al., 2000). Because TRAP uses longer primers than RAPDs, it should have better reproducibility. To test this, we used the same primer pair to amplify fragments from 2 segregating populations of wheat (*T. aestivum* L.): one was a recombinant inbred line (RIL) population, and the other was an F_2 population. Amplification patterns were basically the same, except for different polymorphism levels and polymorphic fragments segregated in the expected Mendelian ratio (1:1 in the RIL and 3:1 in the F_2 populations).

An advantage claimed for both RAPDs and AFLPs over allele-specific PCR markers is that prior sequence information is not needed to generate markers. This advantage was obvious at the time of these inventions. However, the TRAP technique now takes advantage of the availability of sequence information, using the known partial sequence of a candidate gene as the fixed primer and an arbitrary primer to amplify the putative candidate gene regions. Thus, it should be useful in plant genomics research involved in marker-trait association.

Although the TRAP technique was developed with the Li-Cor DNA Sequencer, the general procedures can be easily adapted to other amplified fragment detection systems, such as those using other fluorescence-labeled primers. Actually, the SRAP technique (Li and Quiros, 2001) was developed with the radioisotope-labeling procedure, using silver staining of the sequencing gel to isolate the amplified fragments of interest for sequencing. Therefore, procedures such as these can also be used to detect TRAP fragments when an automatic detecting device is unavailable.

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